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Expression of sex steroid receptors and IGF-1 mRNA in breast tissue — effects of hormonal treatment

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Abstract

The mechanisms behind increased breast tissue proliferation and a possibly increased breast cancer risk in women using hormonal contraception (HC) and hormonal replacement therapy (HRT) are incompletely understood. We analyzed breast tissue from 20 premenopausal and seven postmenopausal women undergoing reduction mammoplasties for estrogen receptor (ER) and progesterone receptor (PR) content as well as mRNA levels for ER, PR and insulin-like growth factor-1 (IGF-1). The receptor values were correlated to IGF-1 mRNA concentrations and levels of steroid and peptide hormones and SHBG. In women using HC, we found significantly lower ER values ($p = 0.02$) but non-significantly lower ER mRNA levels compared to those in naturally cycling women. PR and PR mRNA were no different. Women on HC displayed a higher breast tissue proliferation ($p = 0.05$) expressed as Ki-67, MIB-1 positivity, which was correlated with IGF-1 mRNA ($r_s = 0.82$, $p = 0.04$). Since the concentration of sex steroid receptors in breast tissue is comparatively low and steroid receptors are down-regulated during hormonal treatment, mechanisms other than direct sex steroid receptor action are likely to be present. Our results suggest a role for IGF-1 in the proliferative response of breast tissue during exogenous hormonal treatment.

1. Introduction

Different combinations of estrogens and progestogens are used for contraception and hormonal replacement therapy by many women worldwide. The possibility of an increased risk of breast cancer during such treatment is a subject of lively discussion. While epidemiologic data are conflicting, recent studies and meta-analyses indicate an increased risk in current users and on long-term treatment [1–5].

The basis of the risk associated with hormonal therapies may lie in the regulation of cell proliferation. Within populations of cells in vitro and in vivo, high rates of cell proliferation increase the risk of transformation to the neoplastic phenotype [6]. The breast is a target organ for estrogen and progesterone, but the interaction with receptors, and the mechanisms which regulate breast cell proliferation are still incompletely understood. Such knowledge could help interpret epidemiologic data and evaluate the potential risk of different hormonal therapy regimens.

Hormonal contraceptive treatment has been reported to increase breast epithelial proliferation [7] but suppress estrogen receptor levels in normal breast tissue [8]. In an animal model (surgically postmenopausal cynomolgous macaques), a continuous combined estrogen/progestogen treatment for two and a half years induced markedly increased breast epithelial proliferation, which was apparent despite down-regulation of estrogen and progesterone receptor levels [9].

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Hence mechanisms for proliferation other than estrogen binding to its intranuclear receptor are likely to be present. Several other steroid hormones and peptides, including androgens, prolactin, growth factors like insulin-like growth factor-1 (IGF-1) and also sex hormone binding globulin (SHBG), have been reported to influence proliferation in breast cancer tissue and cancer risk [10–12]. Insulin like growth factors are known mitogens for breast cancer cell lines [13]. Recent in vitro studies have emphasized the importance of interaction between breast epithelium and stroma with autocrine and paracrine growth factors [14].

In the present study, estrogen (ER) and progesterone (PR) receptor expression was quantified and proliferation was assessed in apparently normal breast tissue from women undergoing reduction mammoplasty. Receptor values were correlated to tissue IGF-1 mRNA concentrations and the levels of circulating steroid and peptide hormones and SHBG.

2. Materials and methods

2.1. Study participants

Twenty-seven apparently healthy pre- and postmenopausal women, without any history or symptoms of breast disease, who were subject to reduction mammoplasties accepted to join the study. Venous blood samples were drawn and anamnestic menstrual cycle data were collected on the day of surgery. All premenopausal women (mean age 33.2, range 22–50 years) had regular menstrual cycles (21–35 days). Eight women used hormonal contraception, mostly ethinylestradiol in combination with desogestrel or levonorgestrel, and 12 were non-users. At the time of surgery three were in the follicular and nine were in the luteal phase of the menstrual cycle. Of the postmenopausal women (mean age 55.6, range 49–72 years) three were using HRT, estradiol in combination with levonorgestrel or norethisterone, and four were non-users. The women who did not use hormonal treatment had not taken any sex steroid-containing drugs during the last six months preceding the study.

The study was approved by the local ethics committee and all women gave their informed consent.

2.2. ER and PR determinations

Normal breast tissue biopsies were frozen in liquid nitrogen and kept at −70°C until analyzed. The biopsies, weighing about 0.2 g, were precooled in liquid nitrogen and then pulverized in a Micro-Dismembrator (B. Braun, Biotech International, Melsungen, Germany). The tissue powder was suspended in ice-cold TEM-SH buffer (10 mM TRIS, 1.5 mM EDTA, 5.0 mM Na_2MoO_4, 1 mM monothioglycerol, 0.4 M KCl pH 7.4) at a tissue/buffer ratio of 1:10. Samples were extracted for 30 min in an ice bath, with repeated vortexing every 5 min. The samples were then centrifuged at 192.000 × g for 65 min at +4°C. Protein concentrations were determined according to the method of Lowry et al. [15] and adjusted to 1–4 mg/ml prior to analysis.

For the determinations of ER and PR, EIA Monoclonal kits (Abbott Laboratories, North Chicago, IL., USA) were used according to the instructions of the manufacturer. As the buffer used for the tissue preparations contained KCl, the nuclear pool of receptors was also extracted and thus the total amount of receptors was assayed.

2.3. mRNA determinations

Biopsies from the same patients were also homogenized in an SDS-containing buffer, digested with Proteinase K and subsequently extracted with phenol:-chloroform for preparation of total nucleic acids (TNA). The concentrations of DNA in the TNA samples were measured fluorometrically at the wavelength 458 nm. For measurements of specific mRNA, probes were synthesized in vitro (using reagents supplied by Promega Biotech, Madison, WI, USA) and radiolabelled with ^{35}S-UTP (Amersham, Bucks., UK). Solution hybridization assays were then performed as described earlier [16]. In short, ^{35}S-UTP labelled cRNA was hybridized (20.000 cpm/incubation) at +70°C to the TNA samples. Incubations were performed in duplicate in microcentrifuge tubes (Treff AG, Switzerland) in a total volume of 40 μl containing 0.6 M NaCl, 20 mM Tris HCl, pH 7.5, 4 M EDTA, 0.1% SDS, 0.75 mM dithiothreitol, and 25% formamide. After overnight incubation, each sample was treated for 45 min at 37°C in 1 ml of a solution containing 40 μg RNase A, 0.3 μg RNase T1 (Boehringer–Mannheim, Mannheim, Germany) and 100 μg calf thymus DNA, to digest non-hybridized RNA. Labelled hybrids protected from RNase digestion were precipitated by addition of 100 μl 6 M trichloroacetic acid (TCA) and collected on filters (Whatman GF/C, Maidstone, Kent, UK). The radioactivity of the samples was compared with a standard curve of known amounts of messenger RNA synthesized in vitro and complementary to the probe used. Results are expressed as amol (10^{-18})/μg DNA in the TNA samples.

2.4. Hybridization probes

The probe used for ER mRNA determinations was descended from bcpe1, a full length cDNA of 4838 bases containing the whole open reading frame of the

Table 1
Median values and range for ER in fmol/mg protein, ER mRNA in amol/μg DNA, PR in fmol/mg protein, PR mRNA in amol/μg DNA, IGF-1 in μg/l, IGF-1 mRNA in amol/μg DNA, E_2 in pmol/l, P_4 in nmol/l, SHBG in nmol/l, T in nmol/l, prolactin in μg/l and MIB-1 in percentage of positive cells. Values are given for naturally cycling women, women using HC, postmenopausal women and postmenopausal women using HRT

	Naturally cycling women			Women using HC			Postmenopausal women			Women using HRT		
	n	Mean	Range	*n*	Mean	Range	*n*	Mean	Range	*n*	Mean	Range
ER	11	0.67	0–3.79	7	0.15	0–1.22	3	4.9	0–10.1	3	2.0	1.0–2.7
ER mRNA	12	3.54	0.87–8.69	8	1.93	0.71–5.6	4	3.0	1.7–3.4	3	1.2	1.2–1.89
PR	11	1.95	0–53.7	7	1.75	0–5.4	3	3.1	1.5–7.5	3	7.4	5.2–8.22
PR mRNA	12	1.39	0.36–6.21	8	0.93	0.07–1.96	4	1.2	0.2–3.6	3	0.7	0–0.9
IGF1	12	275.5	159–434	8	300	171–372	2	139	124–154	3	185	90–258
IGF-1 mRNA	12	2.23	0.47–8.79	8	2.78	0–7.85	4	1.3	1.0–2.5	3	0.9	0.8–1.5
E_2	11	301.1	60.1–1554.0	7	107.0	92.4–168.0	2	88.9	61.8–116	3	451	378–663
P_4	11	19.7	3.0–83.8	7	4.3	1.6–6.3	2	1.4	0.6–2.1	3	3.2	3.0–4.1
SHBG	12	44.4	19.4–82.4	8	126.2	12.3–202.2	2	34.6	26.3–42.8	3	66.3	20.6–68.4
T	12	1.45	0.8–1.8	8	1.75	0.6–2.3	2	0.9	0.7–1.1	3	0.6	0.5–0.8
PRL	12	9.6	3.8–37.5	7	14.2	6.1–27.5	2	11.6	10.8–12.5	2	6.5	6.1–6.9
MIB-1 alveoli	12	2	0–15	7	3.0	0–15	1	0.5	0.5	2	0	0–0
MIB-1 ducts	12	2	0.5–15	7	6.0	2–15	3	2.0	0.5–3.0	2	0.75	0.5–1

human estrogen receptor. The cDNA was inserted in a pGEM7zf vector. Restriction of this vector with Bgl II allows the synthesis of a probe corresponding to nucleotides 1470-2062, which encode the C-terminal half of the steroid binding domain (E) and all of domain F.

The probe used for PR mRNA determinations was descended from a full length cDNA containing the whole open reading frame of the human progesterone receptor. The cDNA was inserted in a pGEM3Z vector. Restriction of this vector with Bgl I allows the synthesis of a probe corresponding to nucleotides 2065-2838, which encode the C-terminal part of ligand binding domain (E).

The probe used for IGF-1 mRNA determinations was derived from a 775 bp Rsal–EcoRI fragment cDNA of the human IGF-1. The fragment was cloned into the HincII and EcoRI sites of a bluescript KS vector. Restriction of this vector with Xhol allows the synthesis of a cRNA probe.

2.5. Assessment of proliferation

Formalin-fixed paraffin-embedded tissues were stained using an avidin-biotin-peroxidase method for antigen retrieval from paraffin-embedded tissue [17]. The MIB-1 (Immunotech, Marseille, France) antibody for detection of Ki-67 was used. The Ki-67, MIB-1 monoclonal antibody reacts with a human nuclear antigen which is present in proliferating cells but absent in quiescent cells. Cell cycle analysis showed that the antigen is expressed in G1, S, G2 and mitosis [18]. Immunostained cells were quantified by cell-counting in sections, by an observer blinded to treatments. Epithelial cells lining the alveoli/terminal ducts and major ducts were considered separately. At least 100 cells per slide were counted at three different sites. Cells were graded as unlabelled (0), weakly labelled (1+), moderately labelled (2+), or intensely labelled (3+).

2.6. Serum analyses

Serum concentrations of estradiol 17-β (E_2), progesterone (P_4) and prolactin were determined by radioimmunoassay (RIA) using commercial kits obtained from Diagnostic Products Corp., Los Angeles, CA, USA (E_2, P_4) and Orion Diagnostica OY, Turku, Finland (prolactin). Serum concentrations of testosterone (T) were determined by RIA after extraction with diethyl ether using a commercial kit (“Testosterone Double Antibody”) obtained from Diagnostic Products Corp., Los Angeles, CA, USA. SHBG and IGF-1 were determined by RIA using commercial kits obtained from Eurodiagnostics AB, Malmö, Sweden and Nichols Institute Diagnostics, San Juan Capistrano, CA, USA, respectively. IGF-1 was extracted from serum with acid ethanol prior to analysis. Detection limits, intra- and interassay coefficients of variation were 20 pmol/l, 5% and 5% for E_2; 0.15 nmol/l, 5.8% and 7.2% for P_4; 0.5 μg/l, 3% and 4% for prolactin; 0.13 nmol/l, 8% and 10% for T; 0.05 nmol/l, 4% and 8% for SHBG and 0.6 μg/l, 6% and 10% for IGF-1, respectively.

2.7. Statistical methods

Statistical analysis was performed with the Mann–Whitney U-test and correlations were calculated using

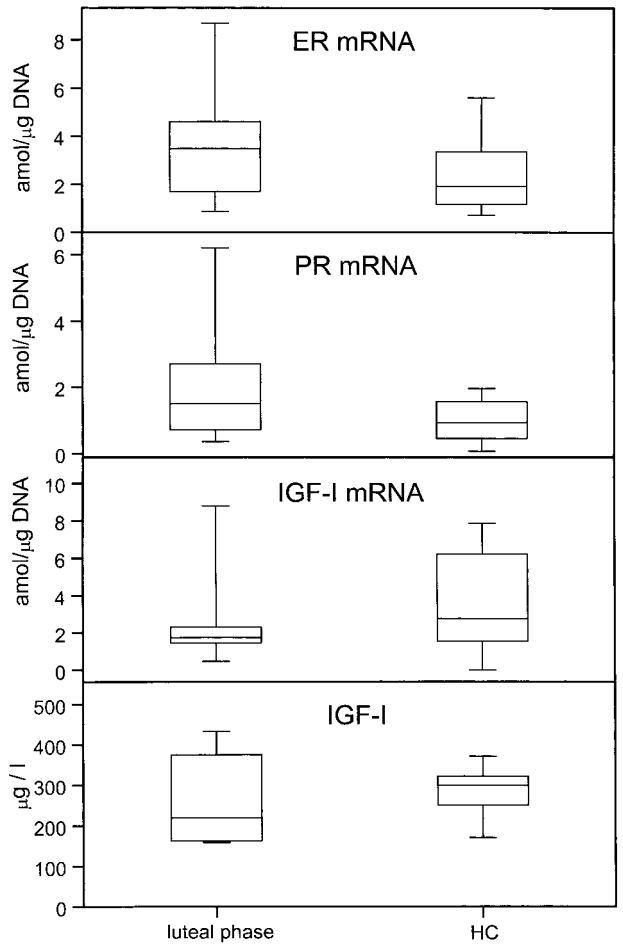

Fig. 1. Box-and-whisker plots representing the median value with 50% of all data falling within the box. The "whiskers" extend to the fifth and 95th percentiles. ER mRNA; PR mRNA; IGF-1 mRNA (amol/µg DNA) and serum IGF-1 (µg/l) in breast tissues from naturally cycling women in luteal phase ($n = 9$) and women on HC ($n = 8$).

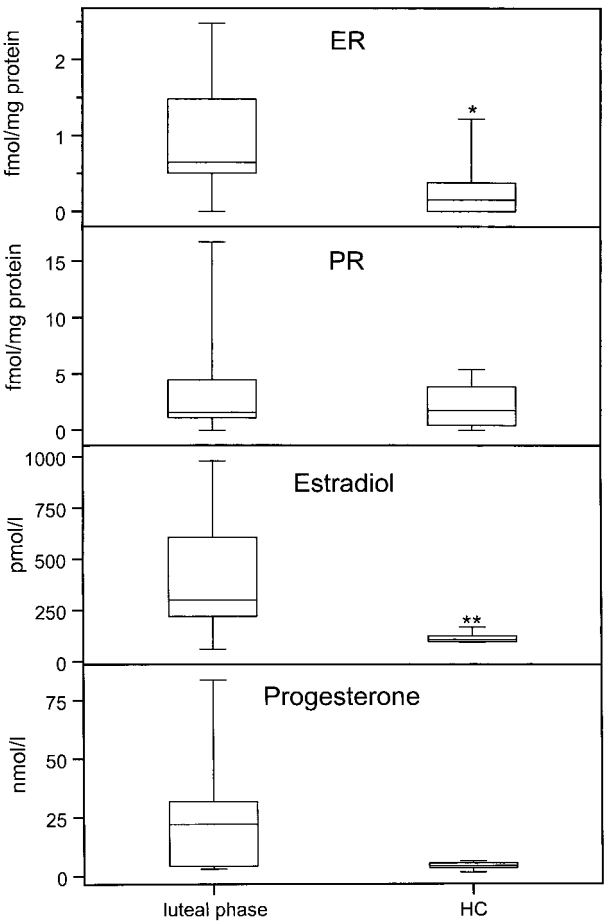

Fig. 2. Box-and-whisker plots representing the median value with 50% of all data falling within the box. The "whiskers" extend to the fifth and 95th percentiles. ER and PR (fmol/mg protein) in breast tissue and serum E_2 (pmol/l) and serum P_4 (nmol/l) from naturally cycling women in luteal phase ($n = 8$) and women on HC ($n = 7$). $^{*}p < 0.05$, $^{**}p < 0.01$.

Spearman's correlation coefficient. Statistical significance was given by $p \leq 0.05$.

3. Results

Results for ER, PR, and IGF-1 expression, breast epithelial cell proliferation and levels of circulating steroid, peptide hormones and SHBG in pre- and postmenopausal women with or without hormonal treatment are given in Table 1. Breast epithelial concentrations of ER were significantly lower ($p = 0.02$) in women on hormonal contraception than in those with spontaneous menstrual cycles. The median ER mRNA concentrations were 1.93 and 3.54 amol/µg DNA, respectively. PR and PR mRNA displayed no apparent differences. The breast tissue of women on hormonal contraception had a higher percentage of MIB-1 positive cells as compared to that of naturally cycling women ($p = 0.05$ for ducts). Among the few postmenopausal women, there was a tendency to lower ER and ER mRNA expression in those on HRT.

Figs. 1 and 2 illustrate a comparison between menstruating women in the luteal phase of the cycle and those on hormonal contraception. During treatment, concentrations of ER, ER mRNA and PR mRNA were lower and serum levels of IGF-1 and tissue concentrations of IGF-1 mRNA higher, than during the luteal phase. However, these differences did not reach statistical significance.

Breast tissue concentrations of IGF-1 mRNA in menstruating women displayed positive correlations with ER mRNA ($r_s = 0.76$, $p = 0.01$) and PR mRNA ($r_s = 0.66$, $p = 0.03$), which were not found in women using hormonal contraceptives. In women on hormonal contraception there was a significant correlation between tissue IGF-1 mRNA and proliferation assessed by percentage of MIB-1 positive cells (alveoli

$r_s = 0.82$, $p = 0.04$). As expected, SHBG levels in serum were higher during hormonal treatment, but otherwise serum markers did not display any differences between groups. Individual serum levels of testosterone showed a significant negative correlation with IGF-1 mRNA expression in breast tissue in women on hormonal contraception ($r_s = -0.76$, $p = 0.04$).

4. Discussion

In the present study we found significantly lower ER values and a significantly higher proliferation in women on hormonal contraceptives, than in the naturally cycling women. These findings are consistent with previous immunohistochemical studies from Williams et al. [7], Battersby et al. [8] and Anderson et al. [19] on breast tissue from women undergoing surgery for benign conditions. PR levels were unchanged or even increased.

During the normal menstrual cycle in fertile women, the highest proliferative activity in breast tissue has been demonstrated in the luteal phase [20]. Immunohistochemical studies have also indicated that estrogen receptors are down-regulated during this phase [8,21].

We found that breast tissue concentrations of specific receptors were low in untreated women also. The concentrations of ER (0–10 fmol/mg protein) and PR (0–50 fmol/mg protein) in breast tissue contrast with those around 200–300 fmol/mg protein and 600–800 fmol/mg protein, respectively found in the uterus by the same technique [22]. In previous immunohistochemical studies, we [23] and others [24] have found a positive receptor staining in only about 5–15% of investigated cells. A similar proportion of stained cells was also observed in a primate model of cynomolgous macaques [9].

In women on hormonal contraception, we found a correlation between IGF-1 mRNA tissue levels and proliferation in breast alveoli. The mean values of IGF-1 and IGF-1 mRNA tended to be higher in these women. Recently high circulating concentrations of IGF-1 were reported as a risk factor for breast cancer in premenopausal women [25]. IGF-1 is known to be a potent mitogen for a range of breast cancer cell lines where it can act in synergy with estradiol to promote growth [26,27].

According to the present data, it seems that not only in breast cancer, but also in the normal breast, exogenous steroid hormone treatment may induce breast cell proliferation by increasing IGF-1 levels (i.e. IGF-1 synthesis). This hypothesis is supported by recent data from a study by Clarke et al. [28] where normal human breast tissue xenografts implanted into athymic nude mice showed increased proliferation and up-regulated IGF-1 mRNA levels after treatment with estradiol.

Alternatively, increased breast proliferation could reflect reduced apoptosis following over-expression of IGF-1 or IGF-1 receptors. IGF-1 inhibited apoptosis during breast involution after lactation in transgenic mice [29]. Furthermore, Streuli et al. [30] found that IGFs suppressed apoptosis in isolated epithelial cells. Elevated serum IGF-1 levels are detected in breast cancer patients [31] and the amount of insulin-like growth factor-1, IGFR-1 is often higher in breast malignancies than in benign tissue [32].

Several other hormones including androgens and progestins have been reported to influence breast tissue proliferation and cancer risk [10,11]. Serum levels of estradiol and progesterone were lower and SHBG levels higher in women on hormonal contraception. Otherwise serum markers displayed no differences between groups. Testosterone levels in serum showed an inverse relationship to breast tissue expression of IGF-1 mRNA during hormonal contraception. Previously, testosterone has been found to suppress proliferation in breast epithelial cells [10,33]. In clinical practice, testosterone and androgenic compounds like danazol are often used to relieve mastalgia and may possibly reduce proliferation [34].

The present data should be interpreted with caution. The size of the clinical material was limited and breast tissue obtained at reduction mammoplasties may not be entirely normal. Still we found a positive correlation between IGF-1 mRNA levels and proliferation in hormonally treated women who had lower ER levels than untreated women. Thus other mechanisms different from the direct sex steroid receptor action are likely to be present and the results suggest a tentative role for IGF-1 in the proliferative response of breast tissue during hormonal treatment.

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